JC09 Rec'd PCT/PTO 03 Aug 2001

FORM PTO-1390 (REV. 11-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY 'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		VOS-013 (107070.120) U.S. APPLICATION NO (If known, see 37 CFR 1.5 09/890752
INTERNATIONAL APPLICATION NO. PCT/DE00/00363	INTERNATIONAL FILING DATE February 4, 2000	PRIORITY DATE CLAIMED February 5, 1999
TITLE OF INVENTION PARTICLES FOR GENE THERAPY		
APPLICANT(S) FOR DO/EO/US Erberhard Hildt		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is attached hereto (required only if not communicated by the International Bureau). (Convenience copy)
   b. [ ] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [X] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [X] A substitute specification. with minor formatting edits
16. [ ] A change of power of attorney and/or address letter.
17. [X] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [ ] Other items or information:

U.S. APPLICATION NO (if known, see 37 CFR 1.5) 09/890752	INTERNATIONAL APPLICATION NO PCT/DE00/00363	ATTORNEY'S DOCKET NUMBER VOS-013

21. [X] The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	17 - 20 =		x **$18.00**	$	
Independent claims	2 - 3 =		x **$80.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$	
SUBTOTAL =				$ 860.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$860.00	
			Amount to be refunded:	$	
			charged:	$	

a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 08-0219 in the amount of $ 860.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-0219 . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO

Ann-Louise Kerner
SIGNATURE

Ann-Louise Kerner
NAME

33,523
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE - DESIGNATED/ELECTED OFFICE

Applicant: Eberhard Hildt et al

Serial No.: 09/890,752
Based on PCT/DE00/00363

International Filing Date: February 4, 2000

U.S. Filing Date: August 3, 2001

Entitled: PARTICLES FOR GENE THERAPY

Attorney Docket No.: VOS-013 (107070.120)

Examiner: TBA

Group Art Unit: TBA

VIA HAND DELIVERY

Box PCT
Assistant Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Applicants respectfully request that the above-referenced patent application be amended before substantive review as follows.

In the Specification:

Please replace the original specification as filed with the Substitute Specification enclosed herewith in accordance with 37 C.F.R. § 1.121(b)(3). The Substitute Specification is submitted in clean form without markings as to amended material and contains numbered paragraphs in accordance with 37 C.F.R. § 1.125(c). Also submitted herewith is a Marked Up Version of the Substitute Specification in accordance with 37 C.F.R. § 1.121(b)(3)(iii) and 1.125(b). A statement in accordance with 37 C.F.R. § 1.125(b) that the Substitute Specification includes no new matter is included herewith.

Please insert the substitute Sequence Listing information (copy enclosed) after the last page of the Substitute Specification (page 15) to replace the original Sequence Listing contained in the Substitute Specification.

In the Drawings:

In accordance with 37 C.F.R. § 1.121(d), proposed changes to Figures 1 and 2 are marked in red for approval by the Examiner on copies of the figures submitted herewith.

In the Claims:

Please cancel claims 1-17 without prejudice or disclaimer of the subject matter contained therein. Please add new claims 18-50 listed below. This list represents all of the claims that will be presently pending.

Pending Claims

18. (New) A particle comprising:

(a) a protein envelope with a fusion protein, the fusion protein comprising a virus protein, a cell permeability-mediating peptide, and a heterologous cell-specific binding site; and

(b) nucleic acid sequences present in the protein envelope, each of the nucleic acid sequences comprising a sequence encoding a virus-specific packaging signal and a sequence encoding a structural gene.

19. (New) The particle of claim 18, wherein the virus protein is derived from the group consisting of adenovirus, adeno-associated virus, vaccinia virus, baculovirus and hepadnavirus.

20. (New) The particle of claim 19, wherein the hepadnavirus is a hepatitis B virus.

21. (New) The particle of claim 18, wherein the virus protein is a surface protein.

22. (New) The particle of claim 22, wherein the surface protein is an LHBs.

23. (New) The particle of claim 18, wherein the virus protein is a core protein.

24. (New) The particle of claim 23, wherein the core protein is an HBcAg.

25. (New) The particle of claim 18, wherein the cell permeability-mediating peptide comprises the amino acid sequence set forth in SEQ ID NO:20.

26. (New) The particle of claim 18, wherein the heterologous cell-specific binding site is RGD.

27. (New) The particle of claim 18, wherein the fusion protein comprises an amino acid sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

28. (New) The particle of claim 18, wherein the fusion protein comprises the amino acid sequence set forth in SEQ ID NO:1.

29. (New) The particle of claim 18, wherein the fusion protein has the amino acid sequence set forth in SEQ ID NO:1.

30. (New) The particle of claim 18, wherein the fusion protein comprises the amino acid sequence set forth in SEQ ID NO:2.

31. (New) The particle of claim 18, wherein the fusion protein has the amino acid sequence set forth in SEQ ID NO:2.

32. (New) A method for the preparation of the particle according to claim 18, wherein the fusion protein contains an LHBs and a heterologous cell-specific binding site, the method comprising:

(a) cotransfecting cells containing a hepatitis B virus genome, wherein the cells do not express LHBs, with a first expression vector coding for a fusion protein, the fusion protein comprising an LHBs and a heterologous cell-specific binding site, and with a second expression vector comprising a virus-specific packaging signal and a structural gene; and

(b) isolating and purifying the particle.

33. (New) A method for the preparation of the particle according to claim 18, wherein the fusion protein comprises an HBcAg, a cell permeability-mediating peptide and a heterologous cell-specific binding site, the method comprising:

(a) cotransfecting cells containing an HBV polymerase with a first expression vector coding for a fusion protein, the fusion protein comprising an HBcAg, a cell permeability-mediating peptide and a heterologous cell-specific binding site, and with a second expression vector comprising a virus-specific packaging signal and a structural gene, and

(b) isolating and purifying the particle.

34. (New) A fusion protein comprising a virus protein, a cell permeability-mediating peptide and a heterologous cell-specific binding site.

35. (New) The fusion protein of claim 34, comprising an amino acid sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

36. (New) The fusion protein of claim 34, comprising the amino acid sequence set forth in SEQ ID NO:1.

37. (New) The fusion protein of claim 34, comprising the amino acid sequence set forth in SEQ ID NO:2

38. (New) The fusion protein of claim 35, wherein the amino acid sequence differs from that set forth in SEQ ID NO:1 or SEQ ID NO:2 by one amino acid.

39. (New) The fusion protein of claim 35, wherein the amino acid sequence differs from that set forth in SEQ ID NO:1 or SEQ ID NO:2 by up to 10%.

40. (New) The fusion protein of claim 35, wherein the amino acid sequence differs from that set forth in SEQ ID NO:1 or SEQ ID NO:2 by up to 20%.

41. (New) A DNA encoding the fusion protein of claim 34.

42. (New) A DNA encoding the fusion protein of claim 35, the DNA comprising a nucleotide sequence selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:4.

43. (New) A DNA encoding the fusion protein of claim 35, the DNA comprising the nucleotide sequence set forth in SEQ ID NO:3.

44. (New) A DNA encoding the fusion protein of claim 35, the DNA comprising the nucleotide sequence set forth in SEQ ID NO:4.

45. (New) The DNA of claim 42, wherein the nucleotide sequence differs from that set forth in SEQ ID NO:3 or SEQ ID NO:4 by one base pair.

46. (New) The DNA of claim 42, wherein the nucleotide sequence differs from that set forth in SEQ ID NO:3 or SEQ ID NO:4 by up to 10%.

47. (New) The DNA of claim 42, wherein the nucleotide sequence differs from that set forth in SEQ ID NO:3 or SEQ ID NO:4 by up to 20%.

48. (New) A DNA encoding the fusion protein of claim 35, wherein the DNA has the nucleotide sequence set forth in SEQ ID NO:3.

49. (New) A DNA encoding the fusion protein of claim 35, wherein the DNA has the nucleotide sequence set forth in SEQ ID NO:4.

50. (New) An expression vector which encodes the DNA of claim 42, 43, 44, 45, 46, 47, 48, or 49.

REMARKS

Claims 1-17 are pending in the application. Claims 1-17 have been cancelled herein without prejudice or disclaimer of the subject matter contained therein for prosecution at a later date. New claims 18-50 have been added herein. Support for these new claims is found in the claims as originally filed as well as the specification at paragraphs 16 and 23. Accordingly, no new matter has been introduced by these amendments. Therefore, after entry of this Preliminary Amendment, claims 18-50 will be pending in the application.

The specification has been amended to correct several obvious typographical errors. In particular, Applicants have amended the text paragraphs (5), (17), (18), (26), (41), and (49), and have amended the sequences in the text to conform to those in the Sequence Listing. Additionally, SEQ ID NOS were added to the specification. Thus, this Amendment does not introduce new subject matter as support is found in the application as filed.

Additionally, the Substitute Sequence Listing now contains SEQ ID NOS:20 and 21 corresponding to the sequences on page 2 of the specification and includes relevant information relating to the U.S. filing date and the inventors. Accordingly, this Amendment does not introduce new subject matter as support is found in the application as filed.

Moreover, Applicants have proposed changes to Figures 1 and 2 to conform the sequences to those in the Sequence Listing as indicated in red on the enclosed sheets. Upon approval by the Examiner, Applicants will file new drawings of Figures 1 and 2 in compliance with 37 C.F.R. § 1.84. Accordingly, these proposed changes do not introduce new subject matter as support is found in the application as filed.

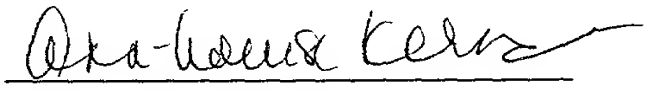
Please charge the fee of $360.00 for extra claims to Deposit Account No. 08-0219.

No additional fees are believe to be due in connection with this correspondence. However, please charge any payments due or credit any overpayments to our Deposit Account No. 08-0219.

Also submitted herewith are a Clean Copy of Substitute Specification, a Marked Up Version of Substitute Specification, a Statement under 37 C.F.R. § 1.125(b)(1), Computer Readable Form of the Sequence Listing, a Paper Copy of the Sequence Listing, a Statement under 37 C.F.R. § 1.821(f), and proposed amendments to Figures 1 and 2.

The Examiner is encouraged to telephone the undersigned in order to expedite the prosecution of the instant application.

Respectfully submitted,
HALE AND DORR LLP



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TO BE FILED

(application with formatting edits)

PARTICLES FOR GENE THERAPY

Eberhard Hildt and Peter Hofschneider

Assignee: Robert Koch Institut

PARTICLES FOR GENE THERAPY

Background of the Invention

The present invention relates to nucleic acid containing particles which specifically bind to cells and can introduce their nucleic acid into these cells. The invention further relates to methods of preparing such particles and means suitable for this purpose as well as the use of the particles in gene therapy.

For gene therapy it is important to have a gene transfer system which is specific, in other words with which desired cells can be reached and genes can be introduced into these cells. In the case of liver cells, this is generally possible with a modified hepatitis B virus (HBV) as a vector, since HBV is specific for liver cells. For other cells, for example fibroblasts, there however exists no gene transfer system which yields satisfactory results.

It is therefore the object of the invention to provide a gene transfer system which is specific, in other words with which desired cells can be reached and genes can be introduced into these cells.

According to the invention, this is achieved by the subject matter in the claims.

Summary of the Invention

The present invention is based on the recognition that nucleic acid-containing particles comprising a fusion protein which includes a virus protein, a cell permeability-mediating peptide, in particular such a peptide as described in the German patent application 198 50 718.6 and a heterologous cell-specific binding site, can bind to corresponding cells and can introduce their nucleic acid into these cells. For example, nucleic acid-containing HBV particles have been made which bind to fibrobalsts and introduce their nucleic acid into these fibroblasts. to this end he exchanged the hepatocyte binding site which is present in the region PreS1, in particular between amino acids 21-47, of the large surface protein of HBV (LHBs) with the $\alpha 5\beta 1$-integrin binding site of fibronectin, wherein the cell permeability-mediating peptide present in the region PreS2 of LHBs remained intact. Furthermore, particles have been made with specificity for fibroblasts by joining the core protein of HBV

(HBcAg) with the α5β1-integrin binding site of fibronectin and the cell permeability-mediating peptide mentioned above. Moreover, the nucleic acid contained in the particles is expressed in the cells.

These findings were used to provide particles including: (a) a protein envelope with a fusion protein comprising a virus protein, a cell permeability-mediating peptide and a heterologous cell-specific binding site; and (b) a nucleic acid present in the protein envelope which comprises sequences for a virus-specific packaging signal and a structural gene.

The term "cell permeability-mediating peptide" includes any peptides capable of mediating a cell permeability for substances, in particular proteins. These are in particular the peptides indicated in the applicant's German patent application 198 50 718.6. Especially preferred is a peptide including the following amino-acid-(DNA)-sequence (SEQ ID NO:20).

P	L	S	S	I	F	S	R	I	G	D	P
CCC	ATA	TCG	TCA	ATC	TTC	TCG	AGG	ATT	GGG	GAC	CCT

The term "cell-specific binding site" includes any binding sites of proteins and other small molecules via which the respective proteins or molecules can bind to cells. Examples of such binding sites are to be found in cytokines and growth factors. They are further to be found in ligands of hormone receptors, neurotransmitter receptors, blood cell surface receptors and integrin receptors. A preferred binding site is the α5β1-integrin binding site of fibronectin. In the following, this binding site is referred to as RGD and includes the amino arginine, glycine and aspartate.

The term "virus" includes DNA and RNA viruses, in particular adenoviruses, adeno-associated viruses, vaccinia viruses, baculoviruses, hepatitis C viruses, hepatitis A viruses, influenza viruses and hepadnaviruses. Examples of the latter are HBV, WHV ("woodchuck hepatitis virus"), GSHV ("ground squirrel hepatitis virus"), RBSHV ("red-bellied squirrel hepatitis virus") DHV ("Pekin duck hepatitis virus") and HHV ("heron hepatitis virus"), wherein HBV is preferred.

The term "virus protein" relates to any protein of a virus mentioned above which can be present in its entirety or partially in a fusion protein together with a cell permeability-mediating peptide and a heterologous cell-specific binding site in the form of a further

peptide. The protein can also already contain the cell permeability-mediating peptide. An example of one such protein is LHBs. This protein is preferred, as are other surface proteins and core proteins, for example HBcAg. The term "heterologous" indicates that the protein does not intrinsically comprise the cell permeability-mediating peptide mentioned above. It can be advantageous when the homologous, i.e. intrinsically present binding site of the protein is switched off. It can be especially advantageous if the homologous binding site is replaced with the heterologous binding site.

The term "nucleic acid" includes RNA and DNA, wherein both can be single stranded and/or double stranded.

The term "virus specific packaging signal" indicates a signal sequence in the above nucleic acids, by means of which the nucleic acids are packaged into the protein envelope of a particle. The signal sequence is specific for an above-mentioned virus. A preferred signal sequence is that of HBV. This is to be found in the HBV DNA and is referred to in the literature as epsilon.

The term "structural gene" includes genes which code for polypeptides (proteins). Examples of such polypeptides are tumor necrosis factors, interferons, interleukins, lymphokines, growth factors, plasma proteins, for example clotting factors and metabolic enzymes, and receptors. In particular the polypeptides can be those which are capable of enhancing the immunogenicity of cells. These can be polypeptides lacking in tumor cells, for example cytokines such as IL-2 and GM-CSF, and co-stimulating molecules such as B7-1, tumor-associated antigens, for example NAGE1, tyrosinases and viral polypeptides, for example E7 from the human papilloma virus and EBNA-3 polypeptides from the Epstein-Barr virus. Furthermore, the polypeptides can be adapter polypeptides, oligomeriztion motifs of a polypeptide, polypeptide fragments of virus envelope polypeptides and hormones. The term "structural gene" further includes antisense oligonucleotides, peptide nucleic acids, consensus sequences for transcription factors and ribozymes.

According to the invention particles containing a fusion protein are preferred, wherein the fusion protein includes an LHBs or fragments thereof and a heterologous binding site, in particular RGD. It is advantageous if the heterologous binding site, for example RGD, is present in place of the homologous binding site. It is especially preferred if the fusion protein

comprises the amino acid sequence of Fig. 1 or an amino acid sequence differing therefrom in one or more amino acids.

Furthermore, particles are preferred which contain a fusion protein which includes a HBcAG, a cell permeability-mediating peptide, for example as indicated in the German patent application 198 50 718.6, in particular with the amino acid sequence given above, and a heterologous binding site, in particular RGD. It is especially preferred if the fusion protein comprises the amino acid sequence of Fig. 2 or an amino acid sequence differing therefrom in one or more amino acids.

The term "an amino acid sequence differing in one or more amino acids" indicates that this amino acid sequence specifies a fusion protein which has comparable elements and functions as the fusion protein in Fig. 1 or figure 2 but which differs from the amino acid sequence of Fig. 1 or Fig. 2 up to 20%, preferably 10%.

A particle according to the invention can be prepared by conventional methods. If the particle contains for example a fusion protein including an LBHs in which the homologous binding site is replaced by a heterologous binding site, in particular RGD, a method containing the following method steps is advantageous: (a) cotransfection of cells coding for hepatitis B virus genome, wherein the cells do not express LHBs, with a first expression vector coding for a fusion protein including an LHBs, in which the homologous binding site is replaced by a heterologous binding site, in particular RGD, and with a second expression vector comprising a virus-specific packaging signal and a structural gene; and (b) isolation and purification of the particle.

If the particle contains a fusion protein including an HBcAg, a cell permeability-mediating peptide according to the German patent application 198 50 718.6, in particular the peptide with the above amino acid sequence, and a heterologous binding site, in particular RGD, then a method including the following steps is advantageous: (a) cotransfection of cells coding for an HBV polymerase with a first expression vector coding for a fusion protein including HBcAg, a cell permeability-mediating peptide according to the German patent application 198 50 718.6, in particular the peptide with the above amino acid sequence, and a heterologous binding site, in particular RGD, and with a second expression vector comprising

a virus-specific packaging signal and a structural gene; and (b) isolation and purification of the particle.

With respect to the terms "expression vector", "cells" and "isolation and purification", reference is made to the explanations below, in particular in the examples. The cells also represent subject matter of the present invention. With respect to the other terms, reference is made to the above explanations.

Further subject matter is a fusion protein including an HBcAg, a cell permeability-mediating peptide and heterologous binding site, in particular RGD. The fusion protein preferably includes the amino acid sequence of Fig. 2 or an amino acid sequence differing therefrom in one or more amino acids.

With respect to the term "an amino acid sequence differing in one or more amino acids", reference is made to the above explanations.

Further subject matter of the present invention is a nucleic acid coding for a fusion protein mentioned above. The nucleic acid can be an RNA or a DNA. Preferably it is a DNA which includes (a) the DNA of Fig. 2 or 2 or a DNA differing therefrom b one or more base pairs, (b) a DNA related to the DNA of (a) by virtue of the degenerate genetic code.

The term "a DNA differing by one or more base pairs" indicates that this DNA codes for a fusion protein which comprises comparable elements and functions as the fusion protein of Fig. 1 or 2, but which differs from the base sequence of Fig. 1 or 2 such that, in the amino acid sequence, a difference of maximum 20%, preferably 10% is present.

A DNA according to the invention can exist as such or in a vector. A DNA according to the invention can in particular exist in an expression vector. Examples of such expression vectors are known to one of ordinary skill in the art. In the case of an expression vector for *E. coli*, these are for example pGEMEX, pUC derivatives, pGEX-2T, pET3b and pQE-8. pY100 and Ycpad1 are examples for expression in yeast, while pKCR, pEFBOS, cDM8, pCEV4, pCDNA3, pKSV10, ;RCMB and pRK5 are examples for the expression in animal cells. The bacculo virus expression vector pAcSGHisNT-A is especially suitable for expression in insect cells.

One of ordinary skill in the art knows suitable cells for the expression of the DNA according to the invention present in an expression vector. Examples of such cells include the E.coli strains HB101, DH1, x 1776, JM101, JM 109, BL21, SG 13009 and M15pRep4, the yeast strain Saccharomyces cerevisiae, the animal cells L, NIH 3T3, FM3A, CHO, COS, Vero, HeLa, Hep62, CCL13 and 293, the insect cells Sf9 and sf21 and the plant cells Lupinus albus.

One of the ordinary skill in the art knows methods and conditions for the transformation or transfectin of cells with an expression vector containing the DNA according to the invention as well as for the cultivation of the cells. He also knows methods for the isolation and purification of the virus protein expressed by the DNA according to the invention.

Further subject matter of the present invention is an antibody directed against the fusion protein mentioned above. Such an antibody can be made by conventional methods. It can be polyclonal or monoclonal. In making it, it is advantageous to immunize animals, in particular rabbits or chickens for a polyclonal antibody and mice for a monoclonal antibody, with the fusion protein. Further "boosters" of the animals with the fusion protein can also take place. The polyclonal antibody can then be obtained from the serum or the egg yolk of the animals. For monoclonal antibodies, the spleen cells of the animals are fused with myeloma cells.

Further subject matter of the present invention is a kit. Such a kit includes one or more of the following components: (a) a fusion protein according to the invention; (b) a DNA according to the invention; (c) an antibody according to the invention; as well as (d) normal adjuvants such as carriers, buffers, solvents, controls, etc.

One or more representatives for each of the individual components can be present. With regard to the individual terms, reference is made to the above explanations.

The present invention provides a gene transfer system which is specific, in other words with which the desired cells can be reached and genes can be introduced into these cells. The cells can be present individually or in a tissue. Furthermore, the cells can be isolated or can be present in the body of an individual. The present invention is therefore suitable for an *ex vivo* or *in vivo* therapy of cells or tissues, respectively. The application of the present invention can be monitored and controlled by antibodies according to the invention.

The present invention therefore represents a major step forward as a way of performing directed modifications to cells or tissues by gene therapy.

Brief Description of the Drawings

Fig. 1 shows the amino acid and DNA sequences of a fusion protein according to the invention which includes an LHBs and the heterologous binding site RGD, wherein the latter replaces the homologous site.

Fig. 2 shows the amino acid and DNA sequences of a fusion protein according to the invention which includes an HBcAg, a cell permeability-mediating peptide of the above amino acid sequence and the heterologous binding site RGD.

Description of the Invention

The present invention is explained by way of the following examples.

Example 1: Preparation of A Particle According To the Invention Which Contains A Fusion Protein Including An LHBs And A Heterologous Binding Site.

(A) Preparation Of An Expression Vector Coding For All HBV-Specific Proteins With The Exception of LHBs

To achieve this, one starts from the plasmid pTKTHBV2 (cf. Will et al., Proc. Natl. Acad. Sci. 82 (1985), 891-895). This contains two copies of the HBV genome. A fragment from ntHBV2821 (first copy) to ntHBV2870 (second copy) is amplified in a first PCR. The forward primer (nt 2821-2855) comprises the following sequence: CCA TAT TCT TGG GAA CAA GAT ATC CAG CAC GGG CC (SEQ ID NO:__) An EcoRV cleavage site is underlined. The triplet ACG between nt 2849-2852 replaces the ATG start codon of LHBs. The backward primer (nt 2877-2845) comprises the following sequence: GGA TTG CTG GTG GAA CAT ATC TGC CCC GTG CTG (SEQ ID NO:___). An EcoRV cleavage site is underlined. The triplet CGT between nt 2852-2849 replaces the natural triplet. CAT. PCR fragments obtained are digested with EcoRV and are purified on a preparative 1% agarose gel. A fragment of about 3.3 kb in size is eluted from the gel and is stored.

In a second PCR, a forward primer comprising an EcoRV cleavage site followed by the subsequent sequence ntHBV2860 (second copy)-2878 (first copy) (CAG CAC GGG GCA GAT ATC TTC CAC CAG CAA TCC (SEQ. ID NO:___), and a backward primer comprising an EcoRV cleavage site followed by the subsequent sequence ntHBV 2830-2810 (GC CCC GTG CTG GAT ATC ATC TTG TTC CCA AGA ATA TGG) (SEQ. ID NO:__) are used. PCR fragments obtained are digested with EcoRV and are purified on a preparative 1% agarose gel. A fragment of the expected size is eluted from the gel and is dephosphorylated. This fragment is used in a ligase reaction with the above fragment of approximately 3.3 kb, wherein the HBV expression vector pTKTHBV2Ldef is obtained. This expression vector codes for all HBV-specific proteins with the exception of LHBs.

(B) Preparation Of An Expression Vector Which Codes For A Fusion Protein Including An LHBs And The Heterologous Binding Site RGD

The fragment ntHBV2990-834 is amplified by PCR starting from the plasmid pTKTHBV2 (cf. above). The 5' primer comprises the following sequence: AAA AGA TCT GGC CGT GGC GAA GGA GCT GGA GCA TTC (SEQ. ID NO:__). This sequence includes a BgIII cleavage site followed by an ATG start codon and the sequence coding for the tripeptide RGD. The PreS1-specific reading from is used. The 3' primer comprises the following sequence: AAA AGA TCT GGT TTA AAT GTA TAC CCA AAG (SEQ. ID NO:__). This sequence includes a BgIII cleavage site. PCR fragments obtained are digested with BgIII and are inserted in the vector pCDNA.3 (Invitrogen), which has been cleaved with BgIII and dephosphorylated, whereby the expression vector pCRGDLHBs is obtained. this expression vector codes for an N-terminally shortened LHBs including the RGD binding site.

(C) Preparation Of An Expression Vector Which Comprises A Structural Gene And A Packaging Signal

A sequence coding for the HBV packaging signal epsilon, for example ntHBV 1840-1914, is amplified by PCR. An EcoRV cleavage site is introduced via the primer used. The sequence of the forward primer reads: CCC GAT ATC ATG TCA TCT CTT GTT CAT GTC CTA (SEQ ID NO: ___). The sequence of the backward primer reads: GGG GAT ATC GGT CGA TGT CCA TGC CCC AAA (SEQ ID: ___). PCR fragments obtained are cleaved with EcoRV and are inserted in the vector pCDNA.3 (cf. above) which has been cleaved with EcoRV and desphosphorylated, whereby the vector pcVPHBV is obtained. This vector contains the HGV-specific packaging signal epsilon.

Starting from the vector pCeGFP (Invitrogen) coding for a "green fluorescent protein" under the control of the CMV promoter, the sequence containing the CMV promoter and the GPF gene is amplified by PCR. The forward primer has the following sequence: GGG GGA TCC CGA TCT ACG GGC CAG ATA TAC GCG TTG (SEQ ID NO: ___). The backward primer has the following sequence: GGG GGA TCC GCG GCC GCT TTA CTT GTA (SEQ ID NO: ___). The primers used each contain a BamHI cleavage site. PCR fragments

obtained are cleaved with BamHI and are inserted into the vector pCVPHBV (Invitrogen) which has been cleaved with BamHI and dephosphorylated, whereby the expression vector pCVPHBVeGPF is obtained. This expression vector contains the HBV-specific packaging signal epsilon, the CMV promoter and a sequence coding for eGFP.

(D) Preparation Of A Packaging Cell

Approximately $0.8x10^6$ HepG2 cells are transfected with 4 µg of pTKTHBV2Ldef (cf. (A)) and 2µg of pCDNA.3 (cf. (B)) by means of lipofection. pCDNA.3 codes for G418 resistance. 2h after transfection, the cells are transferred into a medium containing 700 mg G418/1. G418-resistant clones are subcultured after 14d. The stable integration of pTKTHBV2Ldef is confirmed by means of PCR and southern blots. The expression of the surface protein SHBs from HBV and from HBcAg is confirmed by means of specific antibodies in ELISAS. The packaging cell line HepG2-TKTHBV2Ldef is obtained. This cell line expresses all HBV-specific proteins with the exception of LHBs.

(E) Preparation Of Particles According To The Invention

Approximately $0.8x10^6$ cells of the packaging cell line of (D) are transfected with 3µg of pCRGLHBs (cf. (B)) and 3µg of pCVPHBVeGFP (cf. (B)) by means of lipofection. 72h after transfection, the cells or their supernatants, respectively, are collected and subjected to a PEG precipitation. Subsequently, a CsCI density gradient centrifugation is performed. The particles according to the invention are obtained in pure form. These particles include all HBV-specific proteins with the exception of LHBs, which is replaced by a RGD-LHBs.

Example 2: **Preparation Of A Particle According To The Invention Which Contains A Fusion Protein Including An HBcAg, A Cell Permeability-Mediating Peptide And A Heterologous Binding Site.**

A DNA coding for a cell permeability-mediating peptide (subsequently referred to as ZPP) is used. This DNA has the following sequence: XXX AGA TCT ATG CCC ATA TCG TCA ATC TTC TCG AGG ATT GGG GAC CCT GGA TCC XXX (X denotes any nucleotide) (SEQ ID NO: ___). This sequence has its 5'-end a BgIII cleavage site, followed by an ATG start codon and, at its 3'-end, a BamHI cleavage site. A double stranded DNA molecule

based on the above sequence is cut with BamHI/BgIII and is inserted into the expression vector pCDNA.3 (cf. above), which has been cleaved with BamHI and dephosphorylated, whereby the expression vector pCZPP is obtained.

Furthermore, the expression vector pTKTHBV2 (cf. above) is used to amplify the fragment nt-HBV 1861-2136 by means of PCR. The forward primer includes the following sequence: XXX GGA TCC ACT GTT CAA GCC TCC AAG CTG (SEQ ID NO: ___). This sequence includes a BamHI cleavage site followed by the sequence ntHB 1861-1881. The backward Primer includes the following sequence: XXX GAA TTC TGG ATC TTC CAA ATT AAC ACC CAC CCA (SEQ ID NO: ___). This sequence includes an EcoRI cleavage site followed by the sequence ntHBV 2139-2116. In a second PCR, the fragment ntHBV 2140-2480, which is extended at its 5'-end with the sequence coding for the RGD motif, is amplified. The forward primer includes the following sequence: XXX GAA TTC CGA GGC GAC GCG TCT AGA GAC CTA GTA GTC (SEQ ID NO. ___). This sequence includes and EcoRI cleavage site followed by the sequence coding for the RGD motif, and the sequence ntHV2140-2161. The backward primer includes the following sequence: XXX AAG CTT TCC CCA CCT TAT GAG TCC AAG (SEQ ID NO: ___). This sequence includes a HindIII-cleavage site and the sequence ntHBV 2480-2460.

Fragments obtained from both PCRs are cleaved with EcoRI and are ligated with one another. The litigation product is used as a template for a further PCR, wherein the forward primer from the first litigation product is used as a template for a further PCR, wherein the forward primer from the first PCR is used as a forward primer and the backward primer from the second PCR is used as a backward primer. PCR fragments obtained are cleaved with BamHI/HindIII and are inserted into the vector pCZPP, which has been cleaved with BamHI/HindIII and has been dephosphorylated, whereby the expression vector pCZPPHBcRGC is obtained. This expression vector codes for HBcAG containing the ZPP sequence at the N-terminus and the RGD sequence in the region of the amino acids 79-82.

Furthermore approximately 0.8 x 10^6 HepG2 cells are transfected by means of lipofection with 4 µg of an expression vector coding for HBV polymerase and with 2 µg pCDN3. Here, reference is made to the previous description from example 1 (D). A cell line denoted as HepG2-HBV Pol is obtained.

Approximately 0.8 x 10^6 cells of the cell line HepG2-HBV Pol are transfected with 3µg of pCZPPHBc RGC and 3µg of pCVPHBVeGPF (cf. example 1,B) by means of lipofection. Here, reference is made to the above description of example 1(E). Particles according to the invention are obtained in pure form.

Example 3: **Detection Of The Expression Of A Nucleic Acid Present In Particles According To The Invention In Fibroblasts**

Approximately $1x10^9$ particles according to the invention form example 1(E) or example 2 are solubilized in 100 µl 0.9% saline and are injected into the tail vein of balb/c mice. The soleus-and the tibialis anterior muscle is isolated at 48h after injection and is slowly frozen in a "tissue tag". Cryo-slices are prepared from the frozen preparation and are analyzed under a fluorescence microscope with blue excitation.

A green fluorescence in the fibroblasts is obtained, indicating the expression of the "green fluorescent protein".

Example 4: **Preparation And Purification Of A Fusion Protein According To The Invention**

The fusion protein of Fig. 1 according to the invention is made. To this end, DNA from Fig. 1 is provided at the 5'-end with a BgIII linker and at the 3'-end with a BgIII linker and is subsequently cleaved with the corresponding restriction enzymes. The BgIII/BgIII fragment obtained is inserted into the expression vector pQE8 cleaved with BamHI, so that the expression plasmide pQE8/LHBs is obtained. Such a plasmid codes for a fusion protein made of 6 histidine residues (N-terminus partner) and the fusion protein according to the invention from Fig. 1 (C-terminus partner). pQE8/LHBs is used for the transformation of *E. coli* SG 13009 (cf. Gottesman, S. et. al., J. Bacteriol. 148, (1981), 265-273). The bacteria are cultivated in an LB medium with 100 µg/ml ampicillin and 25 µg/ml kanamycin and are induced for 4h with 60 µM Isopropyl-B-D-Thiogalactopyranoside (IPTG). Lysis of the bacteria is achieved by addition of 6 M guanidine hydrochloride, whereafter chromatography (Ni-NTA-Resin) of the lysate is performed in the presence of 8 M urea according to the directions of the manufacturer (Qiagen) of the chromatography material. The bound fusion protein is eluted in a buffer at pH 3.5. Following neutralization, the fusion protein is

subjected to 18% SDS polyacrylamide gel electrophoresis and is stained with coomassie blue (cf. Thomas, J.O. and Kornberg, R.D., J.Mol.Biol. 149 (1975), 709-733).

It has been found that a fusion protein according to the invention can be made in highly pure form.

Example 5 **Preparation and Detection of An Antibody According to the Invention**

A fusion protein of example 4 according to the invention is subjected to 18% SDS polyacrylamide gel electrophoresis. After staining of the gel with 4 M sodium acetate, a 38 kD band is cut out of the gel and is incubated in phosphate-buffered saline solution. Pieces of the gel are sedimented prior to determination of the protein concentration of the supernatant by SDS polyacrylamide gel electrophoresis and staining with coomassie blue. Animals are immunized with the gel-purified fusion protein as follows:

A) Immunization Protocol for Polyclonal Antibodies In Rabbits

35 µg of gel-purified fusion protein in 0.7 ml PBS and 0.7 ml complete or incomplete Freund's adjuvant are used for each immunization.

Day 0:	1. Immunization (complete Freund's adjuvant)
Day 14:	2. Immunization (incomplete Freund's adjuvant; icFA)
Day 28:	3. Immunization (icFA)
Day 56:	4. Immunization (icFA)
Day 80:	bleeding

The rabbit serum is tested in an immunoblot. To this end, a fusion protein from example 4 according to the invention is subjected to SDS polyacrylamide gel electrophoresis and is transferred to a nitrocellulose filter (cf. Khyse-Andersen, J., J. Biochem. Biophys. Meth. 10, (1984), 203-209). Western blot analysis as described in Bock, C.-T. et al., Virus Genes 8, (1994), 215-229 was performed. To this end, the nitrocellulose filter is incubated for 1 h at 37°C with a first antibody. This antibody is the serum of the rabbit (1:10000 in PBS). After

multiple wash steps with PBS, the nitrocellulose filter is incubated with a second antibody. This antibody is a monoclonal goat anti-rabbit IgG antibody (Dianova) coupled with alkaline phosphatase (1:5000) in PBS. After 30 minutes of incubation at 37°C, multiple wash steps with PBS follow and subsequently the alkaline phosphatase detection reaction is performed with development solution (36 µM 5' bromo-4-chloro-3-indolylphosphate, 400 µM nitroblue tetrazolium, 100 mM Tris-HC1, pH 9.5, 100 mM NaC1, 5 mM $MgCl_2$) at room temperature until bands become visible.

It has been found that polyclonal antibodies according to the invention can be prepared.

B) Immunization Protocol for Polyclonal Antibodies in Chicken

40 µg of gel-purified fusion protein in 0.8 ml PBS and 0.8 ml complete or incomplete Freund's adjuvant are used for each immunization.

Day 0: 1. Immunization (complete Freund's adjuvant)

Day 28: 2. Immunization (incomplete Freund's adjuvant; icFA)

Day 50: 3. Immunization (icFA)

Antibodies are extracted from egg yolk and are tested by western blot. Polyclonal antibodies according to the invention are detected.

C) Immunization Protocol for Monoclonal Antibodies of Mice

12 µg of gel-purified fusion protein in 0.25 ml PBS and 0.25 ml complete or incomplete Freund's adjuvant are used for each immunization; In the fourth immunization, the fusion protein is solubilized in 0.5 ml (without adjuvant).

Day 0: 1. Immunization (complete Freund's adjuvant)

Day 28: 2. Immunization (incomplete Freund's adjuvant; icFA)

Day 56: 3. Immunization (icFA)

Day 84: 4. Immunization (PBS)

Day 87: fusion

Supernatants from hybridomas are tested by Western blot. Monoclonal antibodies according to the invention are detected.

SEQUENCE LISTING

<110> Eberhard Hildt, Prof. Hofschneider

<120> Particles for Gene Therapy

<130> 319-2 US

<140> PCT/DE00/00363
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<220>
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Fusion protein comprising a LHBs and heterologous binding site RGD

<400> 1

Met Gly Arg Gly Asp Gly Ala Gly Ala Phe Gly Leu Gly Phe Thr Pro
1 5 10 15

Pro His Gly Gly Leu Leu Gly Trp Ser Pro Gln Ala Gln Gly Ile Leu
20 25 30

Glu Thr Leu Pro Ala Asn Pro Pro Pro Ala Ser Thr Asn Arg Gln Ser
35 40 45

Gly Arg Gln Pro Thr Pro Leu Ser Pro Pro Leu Arg Asn Thr His Pro
50 55 60

Gln Ala Met Gln Trp Asn Ser Thr Thr Phe His Gln Thr Leu Gln Asp
65 70 75 80

Pro Arg Val Arg Gly Leu Tyr Phe Pro Ala Gly Gly Ser Ser Ser Gly
85 90 95

Thr Val Asn Pro Val Pro Thr Thr Val Ser Pro Ile Ser Ser Ile Phe
100 105 110

Ser Arg Ile Gly Asp Pro Ala Leu Asn Met Glu Asn Ile Thr Ser Gly
115 120 125

Phe Leu Gly Pro Leu Leu Val Leu Gln Ala Gly Phe Phe Leu Leu Thr

130 135 140

Arg Ile Leu Thr Ile Pro Gln Ser Leu Asp Ser Trp Trp Thr Ser Leu
145 150 155 160

Asn Phe Leu Gly Gly Thr Thr Val Cys Leu Gly Gln Asn Ser Gln Ser
165 170 175
Pro Thr Ser Asn His Ser Pro Thr Ser Cys Pro Pro Thr Cys Pro Gly
180 185 190

Tyr Arg Trp Met Cys Leu Arg Arg Phe Ile Ile Phe Leu Phe Ile Leu
195 200 205

Leu Leu Cys Leu Ile Phe Leu Leu Val Leu Leu Asp Tyr Gln Gly Met
210 215 220

Leu Pro Val Cys Pro Leu Ile Pro Gly Ser Ser Thr Thr Ser Thr Gly
225 230 235 240

Pro Cys Arg Thr Cys Thr Thr Pro Ala Gln Gly Thr Ser Met Tyr Pro
245 250 255

Ser Cys Cys Cys Thr Lys Pro Ser Asp Gly Asn Cys Thr Cys Ile Pro
260 265 270

Ile Pro Ser Ser Trp Ala Phe Gly Lys Phe Leu Trp Glu Trp Ala Ser
275 280 285

Ala Arg Phe Ser Trp Leu Ser Leu Leu Val Pro Phe Val Gln Trp Phe
290 295 300

Val Gly Leu Ser Pro Thr Val Trp Leu Ser Val Ile Trp Met Met Trp
305 310 315 320

Tyr Trp Gly Pro Ser Leu Tyr Ser Ile Leu Ser Pro Phe Leu Pro Leu
325 330 335

Leu Pro Ile Phe Phe Cys Leu Trp Val Tyr Ile
340 345

<210> 2
<211> 215
<212> PRT
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Fusion protein comprising a HBcAg, a cell-permeability-mediating polypeptide and heterologous binding site RGD

<400> 2

Met Pro Leu Ser Ser Ile Phe Ser Arg Ile Gly Asp Pro Thr Val Gln
1 5 10 15

Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile Asp Pro
20 25 30

Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu Pro Ser
35 40 45

Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser Ala Leu
50 55 60

Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His His Thr
65 70 75 80

Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr Leu Ala
85 90 95

Thr Trp Val Gly Val Asn Leu Glu Asp Pro Glu Phe Arg Gly Asp Ala
100 105 110

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys
115 120 125

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg
130 135 140

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr
145 150 155 160

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro
165 170 175

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr
180 185 190

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser
195 200 205

Gln Ser Arg Glu Pro Gln Cys
210 215

<210> 3
<211> 663
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
DNA coding for a fusion protein comprising a HBcAg, a cell-permeability-mediating polypeptide and heterologous binding site RGD

<400> 3

atgcccatat cgtcaatctt ctcgaggatt ggggaccctg gatccactac tgttcaagcc 60

tccaagctgt gccttgggtg gctttggggc atggacatcg acccttataa agaatttgga 120

gctactgtgg agttactctc gtttttgcct tctgacttct ttccttcagt acgagatctt 180

ctagataccg cctcagctct gtatcgggaa gccttagagt ctcctgagca ttgttcacct 240

caccatactg cactcaggca agcaattctt tgctgggggg aactaatgac tctagctacc 300

tgggtgggtg ttaatttgga agatccagaa ttccgaggcg acgcgtctag agacctagta 360

gtcagttatg tcaacactaa tatgggccta aagttcaggc aactcttgtg gtttcacatt 420

tcttgtctca cttttggaag agaaaccgtt atagagtatt tggtgtcttt cggagtgtgg 480

attcgcactc ctccagctta tagaccacca aatgccccta tcctatcaac acttccggaa 540

actactgttg ttagacgacg aggcaggtcc cctagaagaa gaactccctc gcctcgcaga 600

cgaaggtctc aatcgccgcg tcgcagaaga tctcaatctc gggaacctca atgttagtat 660

tcc 663

<210> 4
<211> 1047
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
DNA coding for a fusion protein comprising a LHBs and heterologous binding site RGD

<400> 4

atgggccgtg gcgaaggagc tggagcattc gggctgggtt tcaccccacc gcacggaggc 60

cttttggggt ggagccctca ggctcagggc atactacaaa ctttgccagc aaatccgcct 120

cctgcctcca ccaatcgcca gacaggaagg cagcctaccc cgctgtctcc acctttgaga 180

aacactcatc ctcaggccat gcagtggaat tccacaacct ttcaccaaac tctgcaagat 240

cccagagtga gaggcctgta tttccctgct ggtggctcca gttcaggagc agtaaaccct 300

gttccgacta ctgcctctcc cttatcgtca atcttctcga ggattgggga ccctgcgctg 360

aacatggaga acatcacatc aggattccta ggaccccttc tcgtgttaca ggcggggttt 420

ttcttgttga caagaatcct cacaataccg cagagtctag actcgtggtg gacttctctc 480

aattttctag ggggaactac cgtgtgtctt ggccaaaatt cgcagtcccc aacctccaat 540

cactcaccaa cctcctgtcc tccaacttgt cctggttatc gctggatgtg tctgcggcgt 600

tttatcatct tcctcttcat cctgctgcta tgcctcatct tcttgttggt tcttctggac 660

tatcaaggta tgttgcccgt ttgtcctcta attccaggat cctcaaccac cagcacggga 720

ccatgccgaa cctgcatgac tactgctcaa ggaacctcta tgtatccctc ctgttgctgt 780

accaaacctt cggacggaaa ttgcacctgt attcccatcc catcatcctg ggctttcgga 840

aaattcctat gggagtgggc ctcagcccgt ttctcctggc tcagtttact agtgccattt 900

gttcagtggt tcgtagggct ttcccccact gtttggcttt cagttatatg gatgatgtgg 960

tattgggggc caagtctgta cagcatcttg agtccctttt taccgctgtt accaattttc 1020

ttttgtcttt gggtatacat ttaaacc 1047

<210> 5
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 5

ccatattctt gggaacaaga tatccagcac ggggc 35

<210> 6
<211> 33
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 6

ggattgctgg tggaagatat ctgccccgtg ctg 33

<210> 7
<211> 33
<212> DNA
<213> Artificial sequence

<220>

<223> Description of the artificial sequence:
Primer

<400> 7

cagcacgggg cagatatctt ccaccagcaa tcc 33

<210> 8
<211> 38
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 8

gccccgtgct ggatatcatc ttgttcccaa gaatatgg 38

<210> 9
<211> 36
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 9

aaaagatctg gccgtggcga aggagctgga gcattc 36

<210> 10
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 10

aaaagatctg gtttaaatgt atacccaaag 30

<210> 11
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<220>
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Primer

<400> 11

cccgatatca tgtcatctct tgttcatgtc cta 33

<210> 12
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<213> Artificial sequence

<220>
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<210> 13
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<400> 13

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<210> 14
<211> 27
<212> DNA
<213> Artificial sequence

<220>
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<220>
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Primer

<400> 15

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<210> 16
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 16

nnnggatcca ctgttcaagc ctccaagctg 30

<210> 17
<211> 36
<212> DNA
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<220>
<223> Description of the artificial sequence:
Primer

<400> 17

nnngaattct ggatcttcca aattaacacc caccca 36

<210> 18
<211> 39
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 18

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<210> 19
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 19

nnnaagcttt ccccacctta tgagtccaag 30

CLAIMS

1. A particle comprising:

   (a) a protein envelope with a fusion protein comprising a virus protein, a cell permeability-mediating peptide and a heterologous cell-specific binding site; and

   (b) nucleic acid sequences present in the protein envelope, comprising a sequence encoding a virus-specific packaging signal and a sequence encoding a structural gene.

2. The particle according to claim 1, wherein the virus protein is derived from an adenovirus, adeno-associated virus, vaccinia virus, baculovirus or hepadnavirus.

3. The particle according to claim 2, wherein the hepadnavirus is a hepatitis B virus.

4. The particle according to any of claims 1-3, wherein the virus protein is a surface protein.

5. The particle according to claim 4, wherein the surface protein is an LHBs.

6. The particle according to any of claims 1-3, wherein the virus protein is a core protein.

7. The particle according to claim 6, wherein the core protein is an HBcAg.

8. The particle according to any of claims 1-7 wherein the cell permeability-mediating peptide comprises the following amino acid sequence: P L S S I F S R I G D (SEQ ID NO:20)

9. The particle according to any of claims 1-8, wherein the heterologous cell-specific binding site is RGD.

10. The particle according to any of claims 1-9, wherein the fusion protein is that in Fig. 1 (SEQ ID NO___) or 2 (SEQ ID NO:___).

11. A method for the preparation of the particle according to claim 1, wherein the fusion protein contains an LHBs and a heterologous cell-specific binding site, comprising the following method steps:

(a) cotransfection of cells which code for a hepatitis B virus genome, wherein these cells do not express LHBs, with a first expression vector coding for a fusion protein which comprises an LHBs and a heterologous cell-specific binding site, and with a second expression vector comprising a virus-specific packaging signal and a structural gene; and

(b) isolation and purification of the particle.

12. A method for the preparation of the particle according to claim 1, wherein the fusion protein comprises an HBcAg, a cell permeability-mediating peptide and a heterologous cell-specific binding site, comprising the following method steps:

(a) cotransfection of cells coding for an HBV polymerase with a first expression vector coding for a fusion protein which comprises an HBcAg, a cell permeability-mediating peptide and a heterologous cell-specific binding site, and with a second expression vector comprising a virus-specific packaging signal and a structural gene, and

(b) isolation and purification of the particle.

13. A fusion protein, comprising a virus protein, a cell permeability-mediating peptide and a heterologous cell-specific binding site.

14. The fusion protein according to claim 13, comprising the amino acid sequence of Fig. 1 or 2 or an amino acid sequence differing therefrom by one or more amino acids.

15. A DNA which codes for the fusion protein according to claim 13.

16. A DNA which codes for the fusion protein according to claim 14, including,

(a) the DNA from Fig. 1 (SEQ ID NO:___) or 2 (SEQ ID NO:___)or a DNA differing therefrom in one or more base pairs; or

(b) A DNA which is related to the DNA of (a) by virtue of the degenerate genetic code.

17. An expression vector which codes for the DNA according to claim 16.

atgggccgtggcgaaggagctggagcattcgggctgggtttcaccccaccgcacggaggccttttggggtggagccctcaggctca
gggcatactacaaactttgccagcaaatccgcctcctgcctccaccaatcgccagacaggaaggcagcctaccccgctgtctccacct
ttgagaaacactcatcctcaggccatgcagtggaattccaccacctttcaccaaactctgcaagatcccagagtgagaggcctgtatttc
cctgctggtggctccagttcaggagcagtaaaccctgttccgactactgcctctcccttatcgtcaatcttctcgaggattggggaccctg
cgctgaacatggagaacatcactcaggattcctaggacccttctcgtgttacaggcggggtttttcttgttgacaagaatcctcacaata
ccgcagagtctagactcgtggtggacttctctcaattttctagggggaactaccgtgtgtcttggccaaaattcgcagtccccaacctcca
atcactcaccaacctcctgtcctccaacttgtcctggttatcgctggatgtgtctgcggcgttttatcatcttcctcttcatcctgctgctatgc
ctccatcttcttgttggttcttctggactatcaaggtatgttgcccgtttgtcctctaattccaggatcctcaaccaccagcacgggaccatg
ccgaacctgcatgactactgctcaaggaacctctatgtatccctcctgttgctgtaccaaaccttcggacggaaattgcacctgtattccc
atcccatcatcctgggctttcggaaaattcctatgggagtgggcctcagcccgttctcctggctcagtttactagtgccatttgttcagtggt
tcgtagggctttccccactgtttggctttcagttatattggatgatgtggtattgggggccaagtctgtacagcatcttgagtccctttttac
cgctgttaccaattttcttttgtctttgggtatacatttaaacc (SEQ ID NO: _____)

MGRGDGAGAFGLGFTPPHGGLLGWSPQAQGILETLPANPPPASTNRQSGRQPTPLSP
PLRNTHPQAMQWNSTTFHQTLQDPRVRGLYFPAGGSSSGTVNPVPTTVSPISSIFSRIG
DPALNMENITSGFLGPLLVLQAGFFLLTRILTIPQSLDSWWTSLNFLGGTTBCLGQNS
QSPTSNHSPTSCPPTCPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYQGMLPVCPLIPGSS
TTSTGPCRTCTTPAQGTSMYPSCCCTKPSDGNCTCIPIPSSWAFGKFLWEWASARFS
WLSLLVPFVQWFVGLSPTVWLSVIWMMWYWGPSLYSILSPFLPLLPIFFCLWVYI
(SEQ. ID NO: _____)

FIG. 1

atgcccatatcgtcaatcttctcgaggattggggaccctggatccactactgttcaagcctccaagctgtgccttgggtggctttggggc
atggacatcgacccttataaagaatttggagctactgtggagttactctcgtttttgccttctgacttctttccttcagtacgagatcttctaga
taccgcctcagctctgtatcgggaagccttagagtctcctgagcattgttcacctcaccatactgcactcaggcaagcaattctttgctgg
ggggaactaatgactctagctacctgggtgggtgttaatttggaagatccagaattccgaggcgacgcgtctagagacctagtagtcag
ttatgtcaacactaatatgggcctaaagttcaggcaactcttgtggtttcacatttcttgtctcacttttggaagagaaaccgttatagagtatt
tggtgtctttcggagtgtggattcgcactcctccagcttatagaccaccaaatgcccctatcctatcaacacttccggaaactactgttgtta
gacgacgaggcaggtcccctagaagaagaactccctcgcctcgcagacgaaggttctcaatcgccgcgtcgcagaagatctcaatct
cgggaacctcaatgttagtattcc

MPLSSIFSRIGDPTVQASKLCLGWLWGMDIDPYKEFGATVELLSFLPSDFFPSVRDLL
DTASALYREALESPEHCSPHHTALRQAILCWGELMTLATWVGVNLEDPEFRGDASR
DLVVSYVNTNMGLKFRQLLWFHISCLTFGRETVIEYVLSFGVWIRTPPAYRPPNAPIL
STLPETTVVRRRGRSPRRRTPSPRRRRSQSPRRRRSQSREPQC

FIG. 2

PARTICLES FOR GENE THERAPY

The present invention relates to nucleic acid containing particles which specifically bind to cells and can introduce their nucleic acid into these cells. The invention further relates to methods of preparing such particles and means suitable for this purpose as well as the use of the particles in gene therapy.

For gene therapy it is important to have a gene transfer system which is specific, in other words with which desired cells can be reached and genes can be introduced into these cells. In the case of liver cells, this is generally possible with a modified hepatitis B virus (HBV) as a vector, since HBV is specific for liver cells. For other cells, for example fibroblasts, there however exists no gene transfer system which yields satisfactory results.

It is therefore the object of the invention to provide a gene transfer system which is specific, in other words with which desired cells can be reached and genes can be introduced into these cells.

According to the invention, this is achieved by the subject matter in the claims.

The present invention is based on the applicant's recognition that nucleic acid-containing particles comprising a fusion protein which includes a virus protein, a cell permeability-mediating peptide, in particular such a peptide as described in the German patent application 198 50 718.6 and a heterologous cell-specific binding site, can bind to corresponding cells and can introduce their nucleic acid into these cells. The applicant has for example made nucleic acid containing HBV particles which bind to fibroblasts and introduce their nucleic acid into these fibroblasts. To this end he exchanged the hepatocyte binding site which is present in the region PreS1, in particular between amino acids 21-47, of the large surface protein of HBV (LHBs) with the α5β1-integrin binding site of fibronectin, wherein the cell permeability-mediating peptide present in the region PreS2 of LHBs remained intact. Furthermore, he made particles with specificity for fibroblasts by joining the core protein of HBV (HBcAg) with the α5β1-integrin binding site of fibronectin and the cell permeability-mediating peptide mentioned above. Moreover, he recognized that the nucleic acid contained in the particles is expressed in the cells.

According to the invention the applicant's findings are used to provide particles including:

(a) a protein envelope with a fusion protein comprising a virus protein, a cell permeability-mediating peptide and a heterologous cell-specific binding site, and

(b) a nucleic acid present in the protein envelope which comprises sequences for a virus-specific packaging signal and a structural gene.

The term "cell permeability-mediating peptide" includes any peptides capable of mediating a cell permeability for substances, in particular proteins. These are in particular the peptides indicated in the applicant's German patent application 198 50 718.6. Especially preferred is a peptide including the following amino acid-(DNA)-sequence:

P L S S I F S R I G D P

CCC ATA TCG TCA ATC TTC TCG AGG ATT GGG GAC CCT

The term "cell-specific binding site" includes any binding sites of proteins and other small molecules via which the respective proteins or molecules can bind to cells. Examples of such binding sites are to be found in cytokines and growth factors. They are further to be found in ligands of hormone receptors, neurotransmitter receptors, blood cell surface receptors and integrin receptors. A preferred binding site is the $\alpha 5\beta 1$-integrin binding site of fibronectin. In the following, this binding site is referred to as RGD and includes the amino acids arginine, glycine and aspartate.

The term "virus" includes DNA and RNA viruses, in particular adenoviruses, adeno-associated viruses, vaccinia viruses, baculoviruses, hepatitis C viruses, hepatitis A viruses, influenza viruses and hepadnaviruses. Examples of the latter are HBV, WHV ("woodchuck hepatitis virus"), GSHV ("ground squirrel hepatitis virus"), RBSHV ("red-bellied squirrel hepatitis virus") DHV ("Pekin duck hepatitis virus") and HHV ("heron hepatitis virus"), wherein HBV is preferred.

The term „virus protein" relates to any protein of a virus mentioned above which can be present in its entirety or partially in a fusion protein together with a cell permeability-mediating peptide and a heterologous cell-specific binding site in the form of a further peptide. The protein can also already contain the cell permeability-mediating peptide. An example of one such protein is LHBs. This protein is preferred, as are other surface proteins and core proteins, for example HBcAg. The term

"heterologous" indicates that the protein does not intrinsically comprise the cell permeability-mediating peptide mentioned above. It can be advantageous when the homologous, i.e. intrinsically present binding site of the protein is switched off. It can be especially advantageous if the homologous binding site is replaced with the heterologous binding site.

The term "nucleic acid" includes RNA and DNA, wherein both can be single stranded and/or double stranded.

The term "virus specific packaging signal" indicates a signal sequence in the above nucleic acids, by means of which the nucleic acids are packaged into the protein envelope of a particle. The signal sequence is specific for an above-mentioned virus. A preferred signal sequence is that of HBV. This is to be found in the HBV DNA and is referred to in the literature as epsilon.

The term "structural gene" includes genes which code for polypeptides (proteins). Examples of such polypeptides are tumor necrosis factors, interferons, interleukins, lymphokines, growth factors, plasma proteins, for example clotting factors and metabolic enzymes, and receptors. In particular, the polypeptides can be those which are capable of enhancing the immunogenicity of cells. These can be polypeptides lacking in tumor cells, for example cytokines such as IL-2 and GM-CSF, and co-stimulating molecules such as B7-1, tumor-associated antigens, for example MAGE1, tyrosinases and viral polypeptides, for example E7 from the human papilloma virus and EBNA-3 polypeptide from the Epstein-Barr virus. Furthermore, the polypeptides can be adapter polypeptides, oligomeriztion motifs of a polypeptide, polypeptide fragments of virus envelope polypeptides and hormones. The term "structural gene" further includes antisense oligonucleotides, peptide nucleic acids, consensus sequences for transcription factors and ribozymes.

According to the invention particles containing a fusion protein are preferred, wherein the fusion protein includes an LHBs or fragments thereof and a heterologous binding site, in particular RGD. It is advantageous if the heterologous binding site, for example RGD, is present in place of the homologous binding site. It is especially preferred if the fusion protein comprises the amino acid sequence of Fig.1 or an amino acid sequence differing therefrom in one or more amino acids.

Furthermore, particles are preferred which contain a fusion protein which includes an HBcAG, a cell permeability-mediating peptide, for example as indicated in the German patent application 198 50 718.6, in particular with the amino acid sequence given above, and a heterologous binding site,

in particular RGD. It is especially preferred if the fusion protein comprises the amino acid sequence of Fig. 2 or an amino acid sequence differing therefrom in one or more amino acids.

The term "an amino acid sequence differing in one or more amino acids" indicates that this amino acid sequence specifies a fusion protein which has comparable elements and functions as the fusion protein in Fig. 1 or figure 2 but which differs from the amino acid sequence of Fig. 1 or Fig. 2 up to 20%, preferably 10%.

A particle according to the invention can be prepared by conventional methods. If the particle contains for example a fusion protein including an LBHs in which the homologous binding site is replaced by a heterologous binding site, in particular RGD, a method containing the following method steps is advantageous:

(a) cotransfection of cells coding for a hepatitis B virus genome, wherein the cells do not express LHBs, with a first expression vector coding for a fusion protein including an LHBs, in which the homologous binding site is replaced by a heterologous binding site, in particular RGD, and with a second expression vector comprising a virus-specific packaging signal and a structural gene, and

(b) isolation and purification of the particle.

If the particle contains a fusion protein including an HBcAg, a cell permeability-mediating peptide according to the German patent application 198 50 718.6, in particular the peptide with the above amino acid sequence, and a heterologous binding site, in particular RGD, then a method including the following method steps is advantageous:

(a) cotransfection of cells coding for an HBV polymerase with a first expression vector coding for a fusion protein including HBcAg, a cell permeability-mediating peptide according to the German patent application 198 50 718.6, in particular the peptide with the above amino acid sequence, and a heterologous binding site, in particular RGD, and with a second expression vector comprising a virus-specific packaging signal and a structural gene, and

(b) isolation and purification of the particle.

With respect to the terms "expression vector", "cells" and "isolation and purification", reference is made to the explanations below, in particular in the examples. The cells also represent subject matter of the present invention. With respect to the other terms, reference is made to the above explanations.

Further subject matter of the present invention is a fusion protein including an LHBs or fragments thereof and a heterologous binding site, in particular RGD. Preferably the fusion protein includes the amino acid sequence of Fig. 1 or an amino acid sequence differing therefrom in one or more amino acids.

Further subject matter is a fusion protein including an HBcAg, a cell permeability-mediating peptide and heterologous binding site, in particular RGD. The fusion protein preferably includes the amino acid sequence of Fig. 2 or an amino acid sequence differing therefrom in one or more amino acids.

With respect to the term "an amino acid sequence differing in one or more amino acids", reference is made to the above explanations.

Further subject matter of the present invention is a nucleic acid coding for a fusion protein mentioned above. The nucleic acid can be an RNA or a DNA. Preferably it is a DNA which includes the following:

(a) The DNA of Fig. 1 or 2 or a DNA differing therefrom by one or more base pairs, or
(b) a DNA related to the DNA of (a) by virtue of the degenerate genetic code.

The term "a DNA differing by one or more base pairs" indicates that this DNA codes for a fusion protein which comprises comparable elements and functions as the fusion protein of Fig. 1 or 2, but which differs from the base sequence of Fig. 1 or 2 such that, in the amino acid sequence, a difference of maximum 20%, preferably 10% is present.

A DNA according to the invention can exist as such or in a vector. A DNA according to the invention can in particular exist in an expression vector. Examples of such expression vectors are known to one of ordinary skill in the art. In the case of an expression vector for E.coli, these are for example pGEMEX, pUC derivatives, pGEX-2T, pET3b and pQE-8. pY100 and Ycpad1 are

examples for expression in yeast, while pKCR, pEFBOS, cDM8, pCEV4, pCDNA3, pKSV10, pRCMV and pRK5 are examples for the expression in animal cells. The bacculo virus expression vector pAcSGHisNT-A is especially suitable for expression in insect cells.

One of ordinary skill in the art knows suitable cells for the expression of the DNA according to the invention present in an expression vector. Examples of such cells include the E.coli strains HB101, DH1, x1776, JM101, JM 109, BL21, SG 13009 and M15pRep4, the yeast strain Saccharomyces cerevisiae, the animal cells L. NIH 3T3, FM3A, CHO, COS, Vero, HeLa, Hep62, CCL13 and 293, the insect cells Sf9 and Sf21 and the plant cells Lupinus albus.

One of ordinary skill in the art knows methods and conditions for the transformation or transfection of cells with an expression vector containing the DNA according to the invention as well as for the cultivation of the cells. He also knows methods for the isolation and the purification of the virus protein expressed by the DNA according to the invention.

Further subject matter of the present invention is an antibody directed against the fusion protein mentioned above. Such an antibody can be made by conventional methods. It can be polyclonal or monoclonal. In making it, it is advantageous to immunize animals, in particular rabbits or chickens for a polyclonal antibody and mice for a monoclonal antibody, with the fusion protein. Further "boosters" of the animals with the fusion protein can also take place. The polyclonal antibody can then be obtained from the serum or the egg yolk of the animals. For monoclonal antibodies, the spleen cells of the animals are fused with myeloma cells.

Further subject matter of the present invention is a kit. Such a kit includes one or more of the following components:

(a) a fusion protein according to the invention,
(b) a DNA according to the invention,
(c) an antibody according to the invention, as well as
(d) normal adjuvants such as carriers, buffers, solvents, controls, etc.

One or more representatives for each of the individual components can be present. With regard to the individual terms, reference is made to the above explanations.

The present invention provides a gene transfer system which is specific, in other words with which the desired cells can be reached and genes can be introduced into these cells. The cells can be present individually or in a tissue. Furthermore, the cells can be isolated or can be present in the body of an individual. The present invention is therefore suitable for an ex vivo or in vivo therapy of cells or tissues, respectively. The application of the present invention can be monitored and controlled by antibodies according to the invention.

The present invention therefore represents a major step forward as a way of performing directed modifications to cells or tissues by gene therapy.

Short description of the drawings.

Fig. 1 shows the amino acid and DNA sequences of a fusion protein according to the invention which includes an LHBs and the heterologous binding site RGD, wherein the latter replaces the homologous site.

Fig. 2 shows the amino acid and DNA sequences of a fusion protein according to the invention which includes an HBcAg, a cell permeability-mediating peptide of the above amino acid sequence and the heterologous binding site RGD.

The present invention is explained by way of the following examples.

Example 1: Preparation of a particle according to the invention which contains a fusion protein including an LHBs and a heterologous binding site.

(A) Preparation of an expression vector coding for all HBV-specific proteins with the exception of LHBs

To achieve this, one starts from the plasmid pTKTHBV2 (cf. Will et al., Proc. Natl. Acad. Sci. 82 (1985), 891-895). This contains two copies of the HBV genome. A fragment from ntHBV2821 (first copy) to ntHBV2870 (second copy) is amplified in a first PCR. The forward primer (nt 2821-2855) comprises the following sequence: CCA TAT TCT TGG GAA CAA GAT ATC CAG CAC GGG GC. An EcoRV cleavage site is underlined. The triplet ACG between nt 2849-2852 replaces

the ATG start codon of LHBs. The backward primer (nt 2877-2845) comprises the following sequence: GGA TTG CTG GTG GAA GAT ATC TGC CCC GTG CTG. An EcoRV cleavage site is underlined. The triplet CGT between nt 2852-2849 replaces the natural triplet CAT. PCR fragments obtained are digested with EcoRV and are purified on a preparative 1% agarose gel. A fragment of about 3.3 kb in size is eluted from the gel and is stored.

In a second PCR, a forward primer comprising an EcoRV cleavage site followed by the subsequent sequence ntHBV2860 (second copy)-2878 (first copy) (CAG CAC GGG GCA GAT ATC TTC CAC CAG CAA TCC), and a backward primer comprising an EcoRV cleavage site followed by the subsequent sequence ntHBV 2830-2810 (GC CCC GTG CTG GAT ATC ATC TTG TTC CCA AGA ATA TGG) are used. PCR fragments obtained are digested with EcoRV and are purified on a preparative 1% agarose gel. A fragment of the expected size is eluted from the gel and is dephosphorylated. This fragment is used in a ligase reaction with the above fragment of approximately 3.3 kb, wherein the HBV expression vector pTKTHBV2Ldef is obtained. This expression vector codes for all HBV-specific proteins with the exception of LHBs.

(B) Preparation of an expression vector which codes for a fusion protein including an LHBs and the heterologous binding site RGD

The fragment ntHBV2990-834 is amplified by PCR starting from the plasmid pTKTHBV2 (cf. above). The 5' primer comprises the following sequence: AAA AGA TCT GGC CGT GGC GAA GGA GCT GGA GCA TTC. This sequence includes a BglII cleavage site followed by an ATG start codon and the sequence coding for the tripeptide RGD. The PreS1-specific reading frame is used. The 3' primer comprises the following sequence: AAA AGA TCT GGT TTA AAT GTA TAC CCA AAG. This sequence includes a BglII cleavage site. PCR fragments obtained are digested with BglII and are inserted in the vector pCDNA.3 (Invitrogen), which has been cleaved with BglII and dephosphorylated, whereby the expression vector pCRGDLHBs is obtained. This expression vector codes for an N-terminally shortened LHBs including the RGD binding site.

(C) Preparation of an expression vector which comprises a structural gene and a packaging signal

A sequence coding for the HBV packaging signal epsilon, for example ntHBV 1840-1914, is amplified by PCR. An EcoRV cleavage site is introduced via the primer used. The sequence of the

forward primer reads: CCC GAT ATC ATG TCA TCT CTT GTT CAT GTC CTA. The sequence of the backward primer reads: GGG GAT ATC GGT CGA TGT CCA TGC CCC AAA. PCR fragments obtained are cleaved with EcoRV and are inserted in the vector pCDNA.3 (cf. above) which has been cleaved with EcoRV and dephosphorylated, whereby the vector pcVPHBV is obtained. This vector contains the HBV-specific packaging signal epsilon.

Starting from the vector pCeGFP (Invitrogen) coding for a "green fluorescent protein" under the control of the CMV promoter, the sequence containing the CMV promoter and the GFP gene is amplified by PCR. The forward primer has the following sequence: GGG GGA TCC CGA TGT ACG GGC CAG ATA TAC GCG TTG. The backward primer has the following sequence: GGG GGA TCC GCG GCC GCT TTA CTT GTA. The primers used each contain a BamHI cleavage site. PCR fragments obtained are cleaved with BamHI and are inserted into the vector pCVPHBV (Invitrogen) which has been cleaved with BamHI and dephosphorylated, whereby the expression vector pCVPHBVeGFP is obtained. This expression vector contains the HBV-specific packaging signal epsilon, the CMV promoter and a sequence coding for eGFP.

(D) Preparation of a packaging cell line

Approximately $0.8x10^6$ HepG2 cells are transfected with 4 µg of pTKTHBV2Ldef (cf. (A)) and 2 µg of pCDNA.3 (cf. (B)) by means of lipofection. pCDNA.3 codes for G418 resistance. 2h after transfection, the cells are transferred into a medium containing 700 mg G418/l. G418- resistant clones are subcultured after 14d. The stable integration of pTKTHBV2Ldef is confirmed by means of PCR and southern blots. The expression of the surface protein SHBs from HBV and from HBcAg is confirmed by means of specific antibodies in ELISAS. The packaging cell line HepG2-TKTHBV2Ldef is obtained. This cell line expresses all HBV-specific proteins with the exception of LHBs.

(E) Preparation of particles according to the invention

Approximately $0.8x10^6$ cells of the packaging cell line of (D) are transfected with 3µg of pCRGDLHBs (cf. (B)) and 3µg of pCVPHBVeGFP (cf. (B)) by means of lipofection. 72h after transfection, the cells or their supernatants, respectively, are collected and subjected to a PEG precipitation. Subsequently, a CsCl density gradient centrifugation is performed. The particles

according to the invention are obtained in pure form. These particles include all HBV-specific proteins with the exception of LHBs, which is replaced by a RGD-LHBs.

Example 2: Preparation of a particle according to the invention which contains a fusion protein including an HBcAg, a cell permeability-mediating peptide and a heterologous binding site.

A DNA coding for a cell permeability-mediating peptide (subsequently referred to as ZPP) is used. This DNA has the following sequence: XXX AGA TCT ATG CCC ATA TCG TCA ATC TTC TCG AGG ATT GGG GAC CCT GGA TCC XXX (X denotes any nucleotide). This sequence has at its 5`-end a BglII cleavage site, followed by an ATG start codon and, at its 3`-end, a BamHI cleavage site. A double stranded DNA molecule based on the above sequence is cut with BamHI/BglII and is inserted into the expression vector pCDNA.3 (cf. above), which has been cleaved with BamHI and dephosphorylated, whereby the expression vector pCZPP is obtained.

Furthermore, the expression vector pTKTHBV2 (cf. above) is used to amplify the fragment nt-HBV 1861-2136 by means of PCR. The forward primer includes the following sequence: XXX GGA TCC ACT GTT CAA GCC TCC AAG CTG. This sequence includes a BamHI cleavage site followed by the sequence ntHB 1861-1881. The backward Primer includes the following sequence: XXX GAA TTC TGG ATC TTC CAA ATT AAC ACC CAC CCA. This sequence includes an EcoRI cleavage site followed by the sequence ntHBV 2139-2116. In a second PCR, the fragment ntHBV 2140-2480, which is extended at its 5'-end with the sequence coding for the RGD motif, is amplified. The forward primer includes the following sequence: XXX GAA TTC CGA GGC GAC GCG TCT AGA GAC CTA GTA GTC. This sequence includes an EcoRI cleavage site followed by the sequence coding for the RGD motif, and the sequence ntHBV2140-2161. The backward primer includes the following sequence: XXX AAG CTT TCC CCA CCT TAT GAG TCC AAG. This sequence includes a HindIII-cleavage site and the sequence ntHBV 2480-2460.

Fragments obtained from both PCRs are cleaved with EcoRI and are ligated with one another. The ligation product is used as a template for a further PCR, wherein the forward primer from the first PCR is used as a forward primer and the backward primer from the second PCR is used as a backward primer. PCR fragments obtained are cleaved with BamHI/HindIII and are inserted into the vector pCZPP, which has been cleaved with BamHI/HindIII and has been dephosphorylated, whereby the expression vector pCZPPHBcRGC is obtained. This expression vector codes for

HBcAg containing the ZPP sequence at the N-terminus and the RGD sequence in the region of the amino acids 79-82.

Furthermore, approximately $0.8x10^6$ HepG2 cells are transfected by means of lipofection with 4µg of an expression vector coding for HBV polymerase and with 2 µg pCDN3. Here, reference is made to the previous description from example 1 (D). A cell line denoted as HepG2-HBV Pol is obtained.

Approximately $0.8x10^6$ cells of the cell line HepG2-HBV Pol are transfected with 3µg of pCZPPHBc RGC and 3µg of pCVPHBVeGFP (cf. example 1,B) by means of lipofection. Here, reference is made to the above description of example 1(E). Particles according to the invention are obtained in pure form.

Example 3: Detection of the expression of a nucleic acid present in particles according to the invention in fibroblasts

Approximately $1x10^9$ particles according to the invention from example 1(E) or example 2 are solubilized in 100 µl 0.9% saline and are injected into the tail vein of balb/c mice. The soleus- and the tibialis anterior muscle is isolated at 48h after injection and is slowly frozen in a "tissue tag". Cryo-slices are prepared from the frozen preparation and are analyzed under a fluorescence microscope with blue excitation.

A green fluorescence in the fibroblasts is obtained, indicating the expression of the "green fluorescent protein".

Example 4: Preparation and purification of a fusion protein according to the invention

The fusion protein of Fig.1 according to the invention is made. To this end, DNA from Fig.1 is provided at the 5'-end with a BglII linker and at the 3'-end with a BglII linker and is subsequently cleaved with the corresponding restriction enzymes. The BglII/BglII fragment obtained is inserted into the expression vector pQE8 cleaved with BamHI, so that the expression plasmid pQE8/LHBs is obtained. Such a plasmid codes for a fusion protein made of 6 histidine residues (N-terminus partner) and the fusion protein according to the invention from Fig.1 (C-terminus partner). pQE-8/LHBs is used for the transformation of E.coli SG 13009 (cf. Gottesman, S. et al., J. Bacteriol. 148,

(1981), 265-273). The bacteria are cultivated in an LB medium with 100 µg/ml ampicillin and 25 µg/ml kanamycin and are induced for 4h with 60 µM Isopropyl-ß-D-Thiogalactopyranoside (IPTG). Lysis of the bacteria is achieved by addition of 6 M guanidine hydrochloride, whereafter chromatography (Ni-NTA-Resin) of the lysate is performed in the presence of 8 M urea according to the directions of the manufacturer (Qiagen) of the chromatography material. The bound fusion protein is eluted in a buffer at pH 3.5. Following neutralization, the fusion protein is subjected to 18 % SDS polyacrylamide gel electrophoresis and is stained with coomassie blue (cf. Thomas, J.O. and Kornberg, R.D., J.Mol.Biol. 149 (1975), 709-733).

It has been found that a fusion protein according to the invention can be made in highly pure form.

Example 5: Preparation and detection of an antibody according to the invention

A fusion protein of example 4 according to the invention is subjected to 18 % SDS polyacrylamide gel electrophoresis. After staining of the gel with 4 M sodium acetate, a 38 kD band is cut out of the gel and is incubated in phosphate-buffered saline solution. Pieces of the gel are sedimented prior to determination of the protein concentration of the supernatant by SDS polyacrylamide gel electrophoresis and staining with coomassie blue. Animals are immunized with the gel-purified fusion protein as follows:

Immunization protocol for polyclonal antibodies in rabbits

35 µg of gel-purified fusion protein in 0.7 ml PBS and 0.7 ml complete or incomplete Freund's adjuvant are used for each immunization.

Day 0:	1. Immunization (complete Freund's adjuvant)
Day 14:	2. Immunization (incomplete Freund's adjuvant; icFA)
Day 28:	3. Immunization (icFA)
Day 56:	4. Immunization (icFA)
Day 80:	bleeding

The rabbit serum is tested in an immunoblot. To this end, a fusion protein from example 4 according to the invention is subjected to SDS polyacrylamide gel electrophoresis and is transferred to a nitrocellulose filter (cf. Khyse-Andersen, J., J. Biochem. Biophys. Meth. 10, (1984), 203-209). Western blot analysis as described in Bock, C.-T. et al., Virus Genes 8, (1994), 215-229 was performed. To this end, the nitrocellulose filter is incubated for 1 h at 37°C with a first antibody. This antibody is the serum of the rabbit (1:10000 in PBS). After multiple wash steps with PBS, the nitrocellulose filter is incubated with a second antibody. This antibody is a monoclonal goat anti-rabbit IgG antibody (Dianova) coupled with alkaline phosphatase (1:5000) in PBS. After 30 minutes of incubation at 37°C, multiple wash steps with PBS follow and subsequently the alkaline phosphatase detection reaction is performed with development solution (36 µM 5' bromo-4-chloro-3-indolylphosphate, 400 µM nitroblue tetrazolium, 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) at room temperature until bands become visible.

It has been found that polyclonal antibodies according to the invention can be prepared.

Immunization protocol for polyclonal antibodies in chicken

40 µg of gel-purified fusion protein in 0.8 ml PBS and 0.8 ml complete or incomplete Freund's adjuvant are used for each immunization.

Day 0:	1. Immunization (complete Freund's adjuvant)
Day 28:	2. Immunization (incomplete Freund's adjuvant; icFA)
Day 50:	3. Immunization (icFA)

Antibodies are extracted from egg yolk and are tested by western blot. Polyclonal antibodies according to the invention are detected.

Immunization protocol for monoclonal antibodies of mice

12 µg of gel-purified fusion protein in 0.25 ml PBS and 0.25 ml complete or incomplete Freund's adjuvant are used for each immunization; In the fourth immunization, the fusion protein is solubilized in 0.5 ml (without adjuvant).

	Day 0:	1. Immunization (complete Freund's adjuvant)
	Day 28:	2. Immunization (incomplete Freund's adjuvant; icFA)
5	Day 56:	3. Immunization (icFA)
	Day 84:	4. Immunization (PBS)
	Day 87:	fusion

Supernatants from hybridomas are tested by western blot. Monoclonal antibodies according to the
10 invention are detected.

ABSTRACT

Particles for gene therapy

The present invention relates to particles, comprising:

(a) a protein envelope with a fusion protein, which comprises a virus protein, a cell permeability-mediating peptide and a heterologous cell-specific binding site, and

(b) a nucleic acid present in the protein envelope, which comprises the sequence for a virus-specific packaging signal and a structural gene.

The invention further relates to methods for the preparation of such particles and means suitable for this purpose, as well as the use of the particles in gene therapy.

atgggccgtggcgaaggagctggagcattcgggctgggtttcaccccaccgcacggaggccttttggggtggagccctcaggctcagggcat
actacaaactttgccagcaaatccgcctcctgcctccaccaatcgccagacaggaaggcagcctaccccgctgtctccacctttgagaaacact
catcctcaggccatgcagtggaattccacaacctttcaccaaactctgcaagatcccagagtgagaggcctgtatttccctgctggtggctccagt
tcaggagcagtaaaccctgttccgactactgcctctcccttatcgtcaatcttctcgaggattggggaccctgcgctgaacatggagaacatcaca
tcaggattcctaggacccctttctcgtgttacaggcggggtttttcttgttgacaagaatcctcacaataccgcagagtctagactcgtggtggacttc
tctcaattttctaggggaactaccgtgtgtcttggccaaaattcgcagtccccaacctccaatcactcaccaacctcctgtcctccaacttgtcctg
gttatcgctggatgtgtctgcggcgttttatcatcttcctcttcatcctgctgctatgcctcatcttcttgttggttcttctggactatcaaggtatgttgcc
cgtttgtcctctaattccaggatcctcaaccaccagcacgggaccatgccgaacctgcatgactactgctcaaggaacctctatgtatccctcctgt
tgctgtaccaaaccttcggacggaaattgcacctgtattcccatcccatcatcctgggctttcggaaaattcctatgggagtgggcctcagcccgtt
ctcctggctcagtttactagtgccatttgttcagtggttcgtagggctttccccccactgtttggctttcagttatatggatgatgtggtattgggggcca
agtctgtacagcatcttgagtccctttttaccgctgttaccaattttcttttgtctttgggtatacatttaaacc

MGRGDGAGAFGLGFTPPIIGGLLGWSPQAQGILETLPANPPPASTNRQSGRQPTPLSPPLRN
THPQAMQWNSTTFHQTLQDPRVRGLYFPAGGSSSGTVNPVPTTVSPISSIFSRIGDPALNME
NITSGFLGPLLVLQAGFFLLTRILTIPQSLDSWWTSLNFLGGTTVCLGQNSQSPTSNHSPTSC
PPTCPGYRWMCLRRFIIFLFILLLCLIFLLVLLDYQGMLPVCPLIPGSSTTSTGPCRTCTTPAQ
GTSMYPSCCCTKPSDGNCTCIPIPSSWAFGKFLWEWASARFSWLSLLVPFVQWFVGLSPTV
WLSVIWMMWYWGPSLYSILSPFLPLLPIFFCLWVYI

FIG.1

atgcccatatcgtcaatcttctcgaggattggggaccctggatccactactgttcaagcctccaagctgtgccttgggtggctttggggcatggac
atcgacccttataaagaatttggagctactgtggagttactctcgtttttgccttctgacttctttccttcagtacgagatcttctagataccgcctcagct
ctgtatcgggaagccttagagtctcctgagcattgttcacctcaccatactgcactcaggcaagcaattctttgctgggggggaactaatgactctag
ctacctgggtgggtgttaatttggaagatccagaattccgaggcgacgcgtctagagacctagtagtcagttatgtcaacactaatatgggcctaa
agttcaggcaactcttgtggtttcacatttcttgtctcacttttggaagagaaaccgttatagagtatttggtgtctttcggagtgtggattcgcactcct
ccagcttatagaccaccaaatgcccctatcctatcaacacttccggaaactactgttgttagacgacgaggcaggtcccctagaagaagaactcc
ctcgcctcgcagacgaaggtctcaatcgccgcgtcgcagaagatctcaatctcgggaacctcaatgttagtattcc

MPLSSIFSRIGDPTVQASKLCLGWLWGMDIDPYKEFGATVELLSFLPSDFFPSVRDLLDTAS
ALYREALESPEHCSPHHTALRQAILCWGELMTLATWVGVNLEDPEFRGDASRDLVVSYVN
TNMGLKFRQLLWFHISCLTFGRETVIEYLVSFGVWIRTPPAYRPPNAPILSTLPETTVVRRRG
RSPRRRTPSPRRRRSQSPRRRRSQSREPQC

FIG.2

319-2US

DECLARATION AND POWER OF ATTORNEY
(Attorney Docket No: 107070.120)

As below-named inventors, we hereby declare that:

Our residences, post office addresses and citizenship are as stated below next to our names.

We believe that we are the original, and only inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PARTICLES FOR GENE THERAPY

the specification of which (check only one):

[ ] is attached hereto.

[X] was filed as United States Patent Application
Serial No. 09/890,752 on August 3, 2001

[X] was filed as PCT Patent Application Serial No.PCT/DE00/00363 on February 4, 2000
and was amended under PCT Article 19 on _____ (if applicable)

We hereby state that we have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

We acknowledge the duty to disclose information which is material to the patentability of the claims of this application in accordance with Title 37, CFR §1.56(a) and §1.56(b). We also acknowledge the duty to disclose all information which is material to the patentability as defined in 37 CFR §1.56, which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

We hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(e) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by us on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. §119(a)-(d) or 365(b), or 365(a):

COUNTRY (if PCT indicate PCT)	APPLICATION NUMBER	DATE OF FILING	PRIORITY CLAIMED UNDER 35 U.S.C. §119 (YES/NO)
PCT	PCT/DE00/00363	February 4, 2000	Yes
Germany	199 04 800.2	February 5, 1999	Yes

We hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or 365(c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior applications and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATION OR PCT INTERNATIONAL APPLICATION(S) DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. §120 or 365(c):

APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS: (PATENTED, PENDING OR ABANDONED)

POWER OF ATTORNEY: As named inventors, we hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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Dichiara, Peter M.	38,005
Discher, Gregory S.	42,488
Donner, Irah H.	35,120
Goldenberg, Richard A.	38,895
Grieff, Edward D.	38,898
Kennard, Wayne M.	30,271
Kerner, Ph.D., Ann-Louise	33,523
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Park, Keum J.	42,059
Reyes, Jason A.	41,513
Rice, Ph.D., Gretchen A.	37,429
Steinberg, Donald R.	37,241
Superko, Colleen	39,850
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Vallabh, Rajesh	35,761
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Wherefore, we petition that letters patent be granted to us for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe our names to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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Inventor's signature [signature] Date 2.11.01
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Citizenship:

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Inventor's signature [signature] Date 8.11.2001
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SEQUENCE LISTING

<110> Ebernard Hildt, Prof. Hofschneider

<120> Particles for Gene Therapy

<130> 319-2 US

<140> PCT/DE00/00363
<141> 2000-02-04

<150> DE 199 04 800.2
<151> 1999-02-05

<160> 19

<170> PatentIn Ver. 2.1

<210> 1
<211> 347
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Fusion protein comprising a LHBs and heterologous binding site
RGD

<400> 1

Met Gly Arg Gly Asp Gly Ala Gly Ala Phe Gly Leu Gly Phe Thr Pro
1 5 10 15

Pro His Gly Gly Leu Leu Gly Trp Ser Pro Gln Ala Gln Gly Ile Leu
20 25 30

Glu Thr Leu Pro Ala Asn Pro Pro Pro Ala Ser Thr Asn Arg Gln Ser
35 40 45

Gly Arg Gln Pro Thr Pro Leu Ser Pro Pro Leu Arg Asn Thr His Pro
50 55 60

Gln Ala Met Gln Trp Asn Ser Thr Thr Phe His Gln Thr Leu Gln Asp
65 70 75 80

Pro Arg Val Arg Gly Leu Tyr Phe Pro Ala Gly Gly Ser Ser Ser Gly
85 90 95

Thr Val Asn Pro Val Pro Thr Thr Val Ser Pro Ile Ser Ser Ile Phe
100 105 110

Ser Arg Ile Gly Asp Pro Ala Leu Asn Met Glu Asn Ile Thr Ser Gly
115 120 125

Phe Leu Gly Pro Leu Leu Val Leu Gln Ala Gly Phe Phe Leu Leu Thr
130 135 140

Arg Ile Leu Thr Ile Pro Gln Ser Leu Asp Ser Trp Trp Thr Ser Leu
145 150 155 160

Asn Phe Leu Gly Gly Thr Thr Val Cys Leu Gly Gln Asn Ser Gln Ser

```
                    165                 170                 175
Pro Thr Ser Asn His Ser Pro Thr Ser Cys Pro Pro Thr Cys Pro Gly
                180                 185                 190

Tyr Arg Trp Met Cys Leu Arg Arg Phe Ile Ile Phe Leu Phe Ile Leu
            195                 200                 205

Leu Leu Cys Leu Ile Phe Leu Leu Val Leu Leu Asp Tyr Gln Gly Met
    210                 215                 220

Leu Pro Val Cys Pro Leu Ile Pro Gly Ser Ser Thr Thr Ser Thr Gly
225                 230                 235                     240

Pro Cys Arg Thr Cys Thr Thr Pro Ala Gln Gly Thr Ser Met Tyr Pro
                245                 250                 255

Ser Cys Cys Cys Thr Lys Pro Ser Asp Gly Asn Cys Thr Cys Ile Pro
            260                 265                 270

Ile Pro Ser Ser Trp Ala Phe Gly Lys Phe Leu Trp Glu Trp Ala Ser
        275                 280                 285

Ala Arg Phe Ser Trp Leu Ser Leu Leu Val Pro Phe Val Gln Trp Phe
    290                 295                 300

Val Gly Leu Ser Pro Thr Val Trp Leu Ser Val Ile Trp Met Met Trp
305                 310                 315                     320

Tyr Trp Gly Pro Ser Leu Tyr Ser Ile Leu Ser Pro Phe Leu Pro Leu
                325                 330                 335

Leu Pro Ile Phe Phe Cys Leu Trp Val Tyr Ile
            340                 345
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<210> 2
<211> 215
<212> PRT
<213> Artificial sequence

<220>
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      Fusion protein comprising a HBcAg, a cell-permeability-
      mediating polypeptide and heterologous binding site RGD


<400> 2

Met Pro Leu Ser Ser Ile Phe Ser Arg Ile Gly Asp Pro Thr Val Gln
1               5                   10                  15

Ala Ser Lys Leu Cys Leu Gly Trp Leu Trp Gly Met Asp Ile Asp Pro
            20                  25                  30

Tyr Lys Glu Phe Gly Ala Thr Val Glu Leu Leu Ser Phe Leu Pro Ser
        35                  40                  45

Asp Phe Phe Pro Ser Val Arg Asp Leu Leu Asp Thr Ala Ser Ala Leu
    50                  55                  60

Tyr Arg Glu Ala Leu Glu Ser Pro Glu His Cys Ser Pro His His Thr
65                  70                  75                  80

Ala Leu Arg Gln Ala Ile Leu Cys Trp Gly Glu Leu Met Thr Leu Ala
                85                  90                  95
```

Thr Trp Val Gly Val Asn Leu Glu Asp Pro Glu Phe Arg Gly Asp Ala
100 105 110

Ser Arg Asp Leu Val Val Ser Tyr Val Asn Thr Asn Met Gly Leu Lys
115 120 125

Phe Arg Gln Leu Leu Trp Phe His Ile Ser Cys Leu Thr Phe Gly Arg
130 135 140

Glu Thr Val Ile Glu Tyr Leu Val Ser Phe Gly Val Trp Ile Arg Thr
145 150 155 160

Pro Pro Ala Tyr Arg Pro Pro Asn Ala Pro Ile Leu Ser Thr Leu Pro
165 170 175

Glu Thr Thr Val Val Arg Arg Arg Gly Arg Ser Pro Arg Arg Arg Thr
180 185 190

Pro Ser Pro Arg Arg Arg Arg Ser Gln Ser Pro Arg Arg Arg Arg Ser
195 200 205

Gln Ser Arg Glu Pro Gln Cys
210 215

<210> 3
<211> 663
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
DNA coding for a fusion protein comprising a HBcAg, a cell-permeability-mediating polypeptide and heterologous binding site RGD

<400> 3

atgcccatat cgtcaatctt ctcgaggatt ggggaccctg gatccactac tgttcaagcc 60

tccaagctgt gccttgggtg gctttggggc atggacatcg acccttataa agaatttgga 120

gctactgtgg agttactctc gttttttgcct tctgacttct ttccttcagt acgagatctt 180

ctagataccg cctcagctct gtatcgggaa gccttagagt ctcctgagca ttgttcacct 240

caccatactg cactcaggca agcaattctt tgctgggggg aactaatgac tctagctacc 300

tgggtgggtg ttaatttgga agatccagaa ttccgaggcg acgcgtctag agacctagta 360

gtcagttatg tcaacactaa tatgggccta aagttcaggc aactcttgtg gtttcacatt 420

tcttgtctca cttttggaag agaaaccgtt atagagtatt tggtgtcttt cggagtgtgg 480

attcgcactc ctccagctta tagaccacca aatgccccta tcctatcaac acttccggaa 540

actactgttg ttagacgacg aggcaggtcc cctagaagaa gaactccctc gcctcgcaga 600

cgaaggtctc aatcgccgcg tcgcagaaga tctcaatctc gggaacctca atgttagtat 660

tcc 663

<210> 4
<211> 1047
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
DNA coding for a fusion protein comprising a LHBs and heterologous binding site RGD

<400> 4

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cttttggggt ggagccctca ggctcagggc atactacaaa ctttgccagc aaatccgcct 120

cctgcctcca ccaatcgcca gacaggaagg cagcctactc cgctgtctcc acctttgaga 180

aacactcatc ctcaggccat gcagtggaat tccacaacct ttcaccaaac tctgcaagat 240

cccagagtga gaggcctgta tttccctgct ggtggctcca gttcaggagc agtaaaccct 300

gttccgacta ctgcctctcc cttatcgtca atcttctcga ggattgggga ccctgcgctg 360

aacatggaga acatcacatc aggattccta ggacccctcc tcgtgttaca ggcggggttt 420

ttcttgttga caagaatcct cacaataccg cagagtctag actcgtggtg gacttctctc 480

aattttctag ggggaactac cgtgtgtctt ggccaaaatt cgcagtcccc aacctccaat 540

cactcaccaa cctcctgtcc tccaacttgt cctggttatc gctggatgtg tctgcggcgt 600

tttatcatct tcctcttcat cctgctgcta tgcctcatct tcttgttggt tcttctggac 660

tatcaaggta tgttgcccgt ttgtcctcta attccaggat cctcaaccac cagcacgcga 720

ccatgccgaa cctgcatgac tactgctcaa ggaacctcta tgtatccctc ctgttgctgt 780

accaaacctt cggacggaaa ttgcacctgt attcccatcc catcatcctg ggctttcgga 840

aaattcctat gggagtgggc ctcagcccgt ttctcctggc tcagtttact agtgccattt 900

gttcagtggt tcgtagggct ttccccccact gtttggcttt cagttatatg gatgatgtgg 960

tattgggggc caagtctgta cagcatcttg agtccctttt taccgctgtt accaattttc 1020

ttttgtcttt gggtatacat ttaaacc 1047

<210> 5
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 5

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<210> 6
<211> 33
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 6

ggattgctgg tggaagatat ctgccccgtg ctg 33

<210> 7
<211> 33
<212> DNA
<213> Artificial sequence

<220>
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Primer

<400> 7

cagcacgggg cagatatctt ccaccagcaa tcc 33

<210> 8
<211> 38
<212> DNA
<213> Artificial sequence

<220>
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Primer

<400> 8

gccccgtgct ggatatcatc ttgttcccaa gaatatgg 38

<210> 9
<211> 36
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 9

aaaagatctg gccgtggcga aggagctgga gcattc 36

<210> 10
<211> 30
<212> DNA
<213> Artificial sequence

<220>
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Primer

<400> 10

aaaagatctg gtttaaatgt atacccaaag 30

<210> 11
<211> 33
<212> DNA
<213> Artificial sequence

<220>
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Primer

<400> 11

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<210> 12
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 12

ggggatatcg gtcgatgtcc atgccccaaa 30

<210> 13
<211> 36
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 13

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<210> 14
<211> 27
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 14

ggggatccg cggccgcttt acttgta 27

<210> 15
<211> 57
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 15

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<210> 16
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 16

nnnggatcca ctgttcaagc ctccaagctg 30

<210> 17
<211> 36
<212> DNA
<213> Artificial sequence

<220>
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Primer

<400> 17

nnngaattct ggatcttcca aattaacacc caccca 36

<210> 18
<211> 39
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 18

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<210> 19
<211> 30
<212> DNA
<213> Artificial sequence

<220>
<223> Description of the artificial sequence:
Primer

<400> 19

nnnaagcttt ccccaccttа tgagtccaag 30

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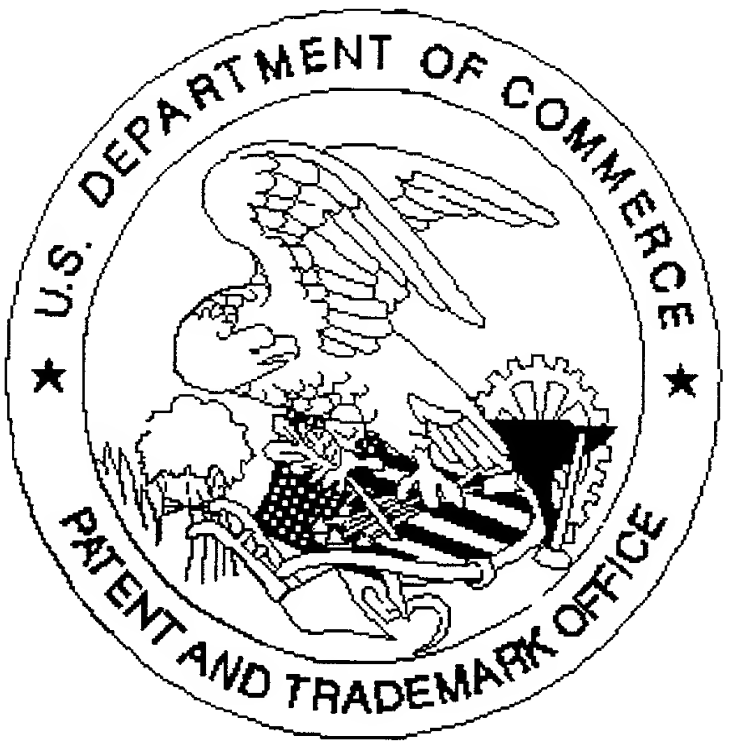

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